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ADVANTAGES OF REAL-TIME PCR AND RD4-PCR AS MOLECULAR METHODS FOR RAPID DETECTION OF BOVINE TUBERCULOSIS IN BULGARIA

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Abstract

Representatives of *Mycobacterium tuberculosis* complex (MTBC) are potential causative agents of tuberculosis in animals and humans, and *Mycobacterium bovis* is considered a major one among domestic and wild ruminants. In the last twenty years, the role of another representative of MTBC – *Mycobacterium caprae*, has been proven in the countries of Central and Southern Europe. Study sample included 121 diagnostic materials from lymph nodes and lungs from cattle, positively and doubtfully PPD tuberculin-reacted, originating from 6 farms belonging to the 4 regions in Northern and Southern Bulgaria. The bacteriological examination showed typical growth for mycobacteria for 110 (90.9%) samples, which was confirmed by qPCR. By RD4-PCR we proved that 102 (92.7%) of the mycobacterial strains were *M. caprae* and the remaining 8 strains (7.3%) were *M. bovis*. This defines *M. caprae* as a dominant species in the etiology of tuberculosis in cattle in Bulgaria.

In conclusion, we confirmed that the application of real-time PCR is an accurate and convenient method for rapid detection of *M. bovis* from cattle in the Bulgarian settings. RD4-PCR provides a sufficiently high differentiation and can be used for first-line typing of *M. bovis* isolates in Bulgaria. This proves the need for the simultaneous application of both methods in the diagnosis of bovine tuberculosis in Bulgaria.

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Introduction. *Mycobacterium tuberculosis* complex (MTBC) is a potential cause of tuberculosis in animals and humans. The main etiological agent of tuberculosis in domestic and wild ruminants is *Mycobacterium bovis*. In the last twenty years, the role of another representative of MTBC has been proven in the countries of Central and Southern Europe – *Mycobacterium caprae*. It is an epidemiological twin of *M. bovis* and evolutionarily older than *M. bovis* [1]. Due to its zoonotic nature, tuberculosis remains a serious challenge for humans and animals in the affected countries [2–5]. Tuberculosis caused by *M. bovis* or *M. caprae* is a rare infection in humans in the EU. According to the latest EFSA report, during the period 2015–2019, the total number of confirmed cases (notification rates) were between 0.03–0.05/100 000 population, mainly among farmers, veterinarians and workers in the meat processing and dairy industry. In 2019, there were 147 confirmed human cases of tuberculosis due to *M. bovis* or *M. caprae* reported by 26 EU Member States. Of these cases, 136 were due to *M. bovis* and 11 were due to *M. caprae* [6].

M. bovis and *M. caprae* have a pronounced tendency to persist in different wildlife hosts, which is a serious prerequisite for maintaining infection in domestic animals. In Europe, vectors of *M. bovis* and *M. caprae* and sources of bovine tuberculosis are most often badgers, free-living European bison, deer, chamois, and wild boar [7–10].

The use of classical (bacteriological and pathomorphological) methods for identification of mycobacteria is still the international gold standard test for laboratory diagnosis of bovine tuberculosis. They are laborious and time-consuming methods and the results take up to three months due to the slow growth rate of mycobacteria.

PCR techniques are widely used for the diagnosis of bTB and have several advantages; they are fast, applied within a few hours, which means rapid diagnosis and efficient monitoring of bovine tuberculosis, and are able to differentiate the mycobacterial species within MTBC.

The aim of the present study was to apply and demonstrate the advantages of real-time PCR for rapid detection of the causative agent of bovine tuberculosis and RD4-PCR for the differentiation of *M. bovis* from *M. caprae*.

Material and methods. ***Diagnostic materials.*** During the period 2020–2022, 121 diagnostic materials from lymph nodes and lungs of cattle that reacted positively and doubtfully to bovine PPD tuberculin were examined. The samples originated from 6 cattle farms with proven tuberculosis from 4 regions in Northern and Southern Bulgaria (Razgrad, Pazardzhik, Stara Zagora and Plovdiv).

Bacteriological examination and DNA extraction. Tissue samples were homogenized and decontaminated with BBL MycoPrep ™Specimen Diges-

tion/Decontamination Kit (BD, USA), kit (BD, USA) containing N-acetyl-L-cysteine and sodium hydroxide NALC-NaOH. Cultivation was performed on Middlebrook 7H9 liquid medium for 14 days and Stonebrink solid medium with pyruvate and PACT at 37 °C for 3–8 weeks. The presence of mycobacteria was confirmed by microscopic observation of Ziehl-Neelsen-stained smears (BD TB Stain Kit ZN for warm staining; Liofilchem, Italy). Total genomic DNA was extracted from liquid bacterial cultures using the Seeplex MTB/NTM ACE Detection Kit (Seegene, USA) according to the manufacturer's protocol.

Real-time PCR (qPCR). Real-time PCR was performed using a commercial Bio-T kit® MTBC (Bioselal, France). For all reactions, MTBC external positive control (EPC), internal positive control (IPC) and negative (dH_2O) controls were added. A total volume of the reaction mix 15 µl containing 10 µL of Master Mix MMMTBC-A and 5 µL DNA was entered into a Rotor-Gene Cycler 6000 (Corbett Research, UK), at the following temperature conditions: preheating 5 min at 95 °C; followed by 40 cycles of two-stage amplification: denaturation 15 s at 95 °C and annealing/extension 15 s at 60 °C.

RD4-PCR. RD4-PCR was performed as described by Ameni et al. [11] with minor revisions: Illustra™ puReTaq Ready-To-Go PCR Beads (GE Healthcare, UK) and the following primers: RD4intF – 5′-ACACGCTGGCGAAGTATAGC-3′, RD4flankR – 5-AAGGCGAACAGATTCAGCAT-3′, RD4falnkF, 5′-CTCGTCGAAGGCCACTAAAG-3′. To each tube was added 25 µl of dH_2O to dissolve the bead, 0.3 µl each primer and 2 µl DNA. Amplification of the target genomic fragments in a total volume of 28 µl was performed using a thermal cycler (Techne TC-412, UK) according to a programme: preheating 15 min/95 °C, followed by 35 cycles: 1 min/95 °C, 1 min/55 °C, 1 min/72 °C and 10 min/72 °C. As a negative control was used ddH_2O, and as positive reference *M. caprae* and *M. bovis* strains. The amplification products were analyzed by gel electrophoresis in 2% GelRed agarose gel and subsequently visualized under UV light.

Results. In the present study, 121 diagnostic materials from lymph nodes and lungs of cattle that reacted positively and doubtfully to bovine PPD tuberculin were studied. The pathoanatomical examination showed the presence of visible lesions in 110 of the studied samples. In the affected lymph nodes we observed tubercles of different sizes and consistency, some encapsulated, others with pronounced calcification or predominant caseous centre. In the lungs of cattle from a farm in the Razgrad region, we found subpleural granulomas of different sizes, with hard, elastic or dough consistency and caseous necrosis (Fig. 1A). Mediastinal LNs were filled with small, encapsulated, whitish, hard nodules (Fig. 1B). The lungs of one cattle from Pazardzhik region were markedly hyperemic, filled with multiple encapsulated granulomas with caseous necrosis (Fig. 1C). Mediastinal LNs were enlarged, protruding, with a whitish cut surface, pronounced cheesy consistency and calcification (Fig. 1D).

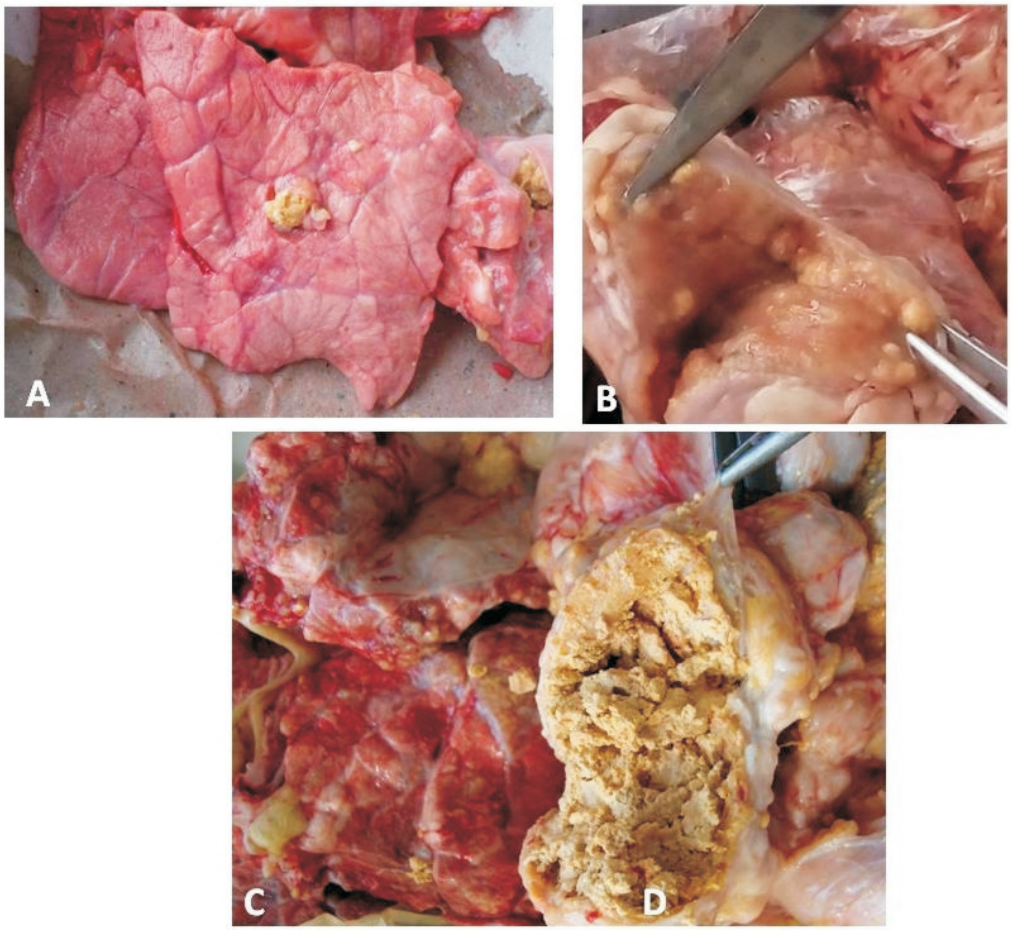

Fig. 1. A and B. Cattle lungs and lymph nodes with the proven presence of *M. bovis*. C and D. Cattle lungs and lymph nodes with proven presence of *M. caprae*

Culture studies on selective liquid and solid media of 110 (90.9%) tissue samples from all organs with visible and no visible lesions (VL) showed the presence of specific growth for mycobacterial MTBC species.

The real-time PCR (qPCR) results are shown in Fig. 2. The obtained amplification curves of the mycobacterial DNAs confirmed their affiliation to the MTBC. All 110 DNA samples gave positive results with Ct values ranging between 9 and 35 cycles. The increase in fluorescence was observed after the 9th cycle, when the sufficiently amplified product had accumulated to produce a detectable fluorescence signal using control from *M. bovis* and *M. caprae*. The negative PCR control shows a completely flat plot due to the absence of a fluorescence signal.

The study panel was further tested by RD4-PCR for differentiation of the members in the MBTC. The results showed the presence of two species of my-

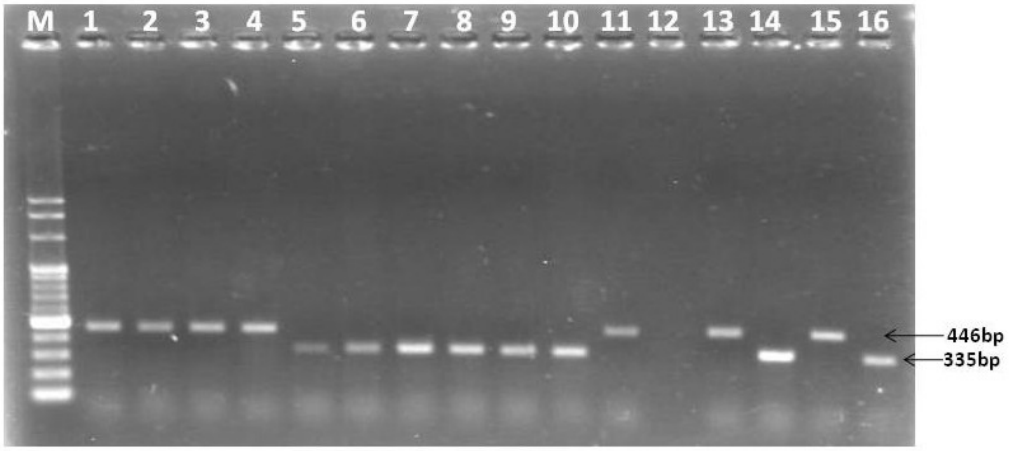

Fig. 2. Real-time amplification curves for M. *caprae* and *M. bovis*

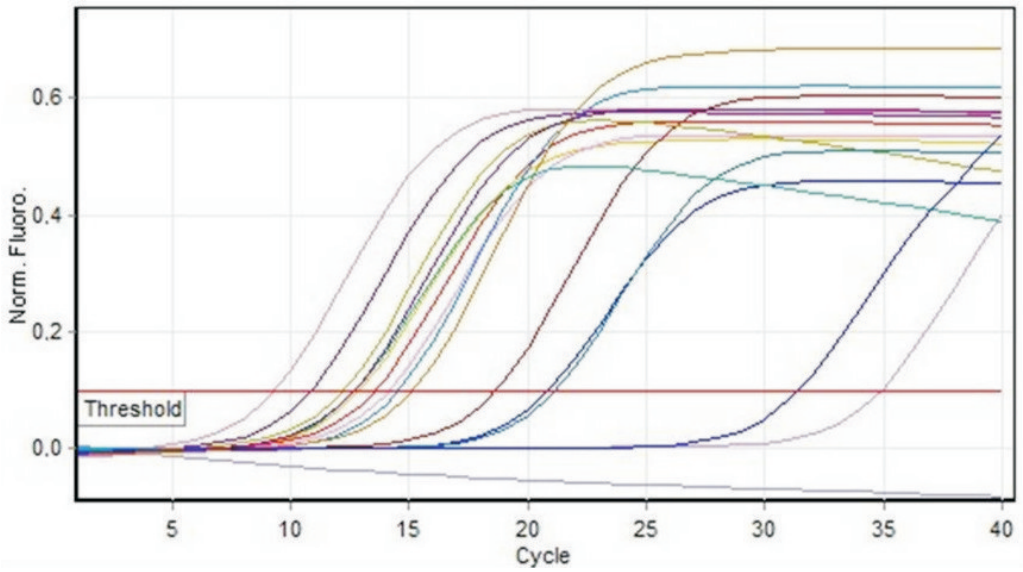

Fig. 3. RD4-PCR: M-100bp, 1–4, 11, 13 – *M. bovis*, 5–10, 14 – *M. caprae*, 12 – negative control (dH_2O), 15 – positive control *M. bovis*, 16 – positive control *M. caprae*

cobacteria – *M. bovis* (8 strains, 7.3%) and *M. caprae* (102 strains, 92.73%). The strains with RD4 deletions in the genome and PCR product amplified at 446 bp were positive for *M. bovis*, while at 335 bp they were positive for *M. caprae* (Fig. 3). *M. bovis* strains originated from farms in Northeastern Bulgaria (Razgrad), and the remaining strains were isolated from farms in Southern Bulgaria (Pazardzhik, Plovdiv and Stara Zagora).

Discussion. Natural *Mycobacterium bovis* infections most commonly affect the lymph nodes (LNs) in the chest, especially the mediastinal, retropharyngeal, and tracheobronchial, and lungs in 30–50% of infected animals [12–14]. In the macroscopic examination of the lymph nodes from slaughtered cattle, we observed LNs with tubercles at different stages of development.

In most of the affected LNs (mostly mediastinal) we found single or disseminated small, encapsulated tubercles (Fig. 1B). In some LNs, the granulomas had pronounced necrosis or calcification, as found in both animals with affected lungs (Fig. 1D). Probably the annual tuberculin testing of cattle and the removal of positive reagents does not allow the infection to develop and it remains localized in the regional lymph nodes. This process is determined by HUCHZERMEYER et al. [15] as the "*primary incomplete tuberculosis complex*" (Fig. 1B). The obtained pathological changes in the lymph nodes can be categorized as LNs with granulomas in stages I and II (*initial*), LNs with caseous necrosis and calcification in stage III (*necrotic*) and LNs in stage IV of development (*necrotic and mineralized*) (Fig. 1A, C, D), which is also proven by WANGOO et al. [16]. We believe that the last two stages are an advanced tuberculosis process (*chronic organ tuberculosis*) typical of adult cattle.

Animal tuberculosis is now a significant problem in many EU countries. According to current legislation (64/432/ECC), the official tests for determining the health status of herds are the intradermal tuberculin test, and the microbiological examination of bovine tissues [17]. However, the skin tuberculin test often causes false-positive reactions in cattle, buffalo and other animals due to interference from non-tuberculous mycobacteria [18]. Although a bacteriological test is the "gold standard" for the detection of tuberculosis in slaughtered animals, other methods for detecting MTBC members directly from tissue samples have been developed in the last decade to accelerate the diagnosis. Different RT-PCR studies showed that the real-time PCR and conventional PCR targeting the RDs regions of the genome are fast and accurate methods comparable to culture detection and have great potential for detecting infected live animals and confirming cases in slaughtered animals [18]. Our results from qPCR in the studied samples are completely comparable to the results obtained from the bacteriological test. Other authors have reported similar results and demonstrated the usefulness of qPCR in diagnostic laboratory practice, even if it cannot be used for differentiation between members of the MBTC [17–19].

The application of RDs-PCR, detects the presence or absence (deletions) of large DNA fragments and allows the differentiation of the strains within the MTBC [20]. RD4-PCR is a specific PCR that uses the detection of RD4 deletions in the genome of *M. bovis* as a genotypic marker to confirm the species of *M. bovis* in MTBC [11]. In our study, this PCR successfully subdivided the samples into 102 *M. caprae* and 8 *M. bovis* strains. The observed distribution of *M. caprae* may be indicative of a geographical-type appearance in Bulgaria. This fact is explained

by the serious eradication programme over the years for the rehabilitation of the affected tuberculosis farms, which necessitated a significant change in the composition of herds through imports mainly from Central European countries, where *M. caprae* is widespread. Domogalla et al. [21] found three different variants of RD4 in *M. caprae* alpine goat isolates. This indicates that the RD4 region in the genome of *M. caprae* is quite heterogeneous and allows the identification of various genotypes of *M. caprae.* This may indicate the geographical distribution of the strains and help to clarify the transmission routes of *M. caprae* between wildlife and cattle.

The ability to identify and differentiate different mycobacterial species is important for the epidemiological studies for determining of the strategies for the control and prevention of bovine tuberculosis in our country. The established variability of the RD4 region in the genomes of *M. caprae* suggests the need for future more extensive and in-depth study of strains isolated from cattle and other potential hosts in domestic and wild animals, which would help to permanently limit and eradicate the disease in Bulgaria.

In conclusion, our results confirmed the advantages of qPCR and RD4-PCR as more sensitive and rapid methods, which successfully demonstrated their usefulness for routine laboratory diagnostics of bovine tuberculosis in the Bulgarian settings. RD4-PCR provides sufficiently high differentiation and may be used for first-line typing. This proves the need for the simultaneous application of both methods in the diagnosis of bovine tuberculosis in Bulgaria.

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